Facilitation of L-type calcium currents by diastolic depolarization in cardiac cells: impairment in heart failure

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Received 5 November 1999; accepted 19 April 2000

Abstract

Objective: Decay kinetics of the voltage-gated L-type Ca\(^{2+}\) current (I\(_{\text{CaL}}\)) control the magnitude of Ca\(^{2+}\) influx during the cardiac action potential. We investigated the influence of changes in diastolic membrane potential on I\(_{\text{CaL}}\) decay kinetics in cardiac cells.

Methods: Cells were isolated enzymatically from rat ventricles, human right atrial appendages obtained during corrective heart surgery and left ventricles from end-stage failing hearts of transplant recipients. The whole-cell patch-clamp technique was used to evoke I\(_{\text{CaL}}\) by a 100-ms depolarizing test pulse to \(-10\) mV. Conditioning potentials between \(-80\) and \(-50\) mV were applied for 5 s prior to the test pulse.

Results: Depolarizing the cells between \(-80\) and \(-50\) mV prior to the test pulse slowed the early inactivation of I\(_{\text{CaL}}\) both in rat ventricular and human atrial cells. This slowing resulted in a significant increase of Ca\(^{2+}\) influx. This type of facilitation was not observed when the sarcoplasmic reticulum (SR) Ca\(^{2+}\) content was depleted using ryanodine which reduced the rate of inactivation of I\(_{\text{CaL}}\), or when Ba\(^{2+}\) replaced Ca\(^{2+}\) as the permeating ion. Facilitation was favored by intracellular cAMP-promoting agents that, in addition to increasing current peak amplitude, enhanced the fast Ca\(^{2+}\)-dependent inactivation of I\(_{\text{CaL}}\). Facilitation was impaired in atrial and ventricular human failing hearts.

Conclusion: Decay kinetics of I\(_{\text{CaL}}\) are regulated by the diastolic membrane potential in rat and human cardiomyocytes. This regulation, which associates slowing of I\(_{\text{CaL}}\) inactivation with reduced SR Ca\(^{2+}\) release and underlies facilitation of Ca\(^{2+}\) channels activity, may have profound physiological relevance for catecholamines enhancement of Ca\(^{2+}\) influx. It is impaired in failing hearts, possibly due to lowered SR Ca\(^{2+}\) release.

Keywords: Adrenergic (ant)agonists; Ca-channel; Heart failure; Membrane potential; Myocytes; Serotonin (5HT)

1. Introduction

The excitation–contraction coupling and contractile force of the myocardium depend upon initial activation of transmembrane L-type Ca\(^{2+}\) channels [1,2]. These channels are closed at negative resting membrane potentials and are gated primarily by membrane depolarization during the action potential. Their activity is also regulated by various transmitters, hormones and intracellular messengers. Best known, \(\beta\)-adrenergic stimulation increases intracellular cAMP which in turn activates protein kinase A and, ultimately, leads to increased open channel probability via a phosphorylation process [3,4]. Ca\(^{2+}\) channel antagonists, which bind to the Ca\(^{2+}\) channel protein and decrease its functional activity, and \(\beta\)-adrenergic blockers, which lower intracellular cAMP, represent two distinct classes of therapeutic agents used to decrease Ca\(^{2+}\) influx in the myocardium [4,5].

In addition to peak current amplitude generated by channel opening, the kinetics of inactivation of the L-type Ca\(^{2+}\) channels are important to determine the duration and, thereby, the amount of Ca\(^{2+}\) entry during membrane depolarization [2–5]. For example, an increase in the frequency of activation of Ca\(^{2+}\) channels induces an increase of Ca\(^{2+}\) entry resulting from a slowing of the decay of I\(_{\text{CaL}}\) both in animal and human cardiomyocytes [6–15]. This mechanism, referred to as frequency-dependent facilitation, occurs over a wide range of frequencies (0.5–5 Hz) corresponding to heart rates encountered in pathophysiology and could be involved in the force–
frequency relation of the healthy myocardium [13,14]. We have recently shown that moderate depolarization in the cell diastolic membrane potential also promotes facilitation of \( I_{\text{cat}} \) in rat ventricular cells [9,11]. In the present study, we investigated regulation of this process by \( \beta \)-adrenergic receptors stimulation and intracellular cAMP, a major regulatory pathway of cardiac function. In addition, we bring evidence for the presence of depolarization-induced facilitation in human cardiomyocytes. We also investigated this regulation in cells isolated from end-stage failing human hearts.

2. Methods

2.1. Rat ventricular myocytes

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Cells were enzymatically isolated from 6- to 10-week old Wistar Kyoto rats as described before [9,11]. Briefly, rats were anesthetized with 30 mg/kg of pentobarbital sodium injected intraperitoneally. The hearts were quickly removed and perfused retrogradely through the aorta (at 37°C) with a Ca\(^{2+}\)-free solution containing (in mmol/l): NaCl (112), KCl (6), MgCl\(_2\) (2), NaHCO\(_3\) (4), KH\(_2\)PO\(_4\) (1.5), HEPES (25), glucose (5.8), penicillin G (60 \( \mu \)g/ml), streptomycin (100 \( \mu \)g/ml) and 0.002% phenol red indicator (pH adjusted at 7.5 with KOH). This step was followed by 20-min perfusion of the same solution supplemented with Ca\(^{2+}\) (10 \( \mu \)mol/l) and 0.8 mg/ml type A collagenase (Boehringer Mannheim). Following perfusion, the ventricles were chopped and incubated in fresh enzymatic solution for 5 min (at 37°C). The tissue samples were rinsed with (in mmol/l): Choline Cl (119), KH\(_2\)PO\(_4\) (1.5), MgCl\(_2\) (1.7), HEPES (25), glucose (5.8), sucinate (5), pyruvic acid (10), creatine (5), and BSA (1 mg/ml), penicillin (60 \( \mu \)g/ml), and 0.002% phenol red indicator (pH adjusted to 7.4 with TEA-OH). The cells were then mechanically dispersed and stored in this solution at 4°C until electrophysiological recordings.

2.2. Human atrial and ventricular cells

Myocytes were isolated as described before [13,16–18]. Briefly, fragments of the right atrial appendage were obtained during open heart surgery (before cardio-pulmonary bypass) from ten patients aged 48 to 79 years in accordance with the institutional guidelines for human subject research of the University Hospital. The clinical diagnosis was aortic or mitral disease (stenosis or insufficiency) or coronary artery disease. Most patients had previously received medication comprising Ca\(^{2+}\) channel antagonists and/or \( \beta \)-adrenergic blockers and/or angiotensin-converting enzyme inhibitors until surgery. During anesthesia, all patients received a benzodiazepine, \( \beta \)-turbocurare, morphine, pentobarbital and antibiotics. Samples of the left ventricle and right atrium were obtained from transplant recipients (aged 44 to 56 years) with either ischemic (two patients) or dilated cardiomyopathy (one patient). These patients had end-stage heart failure (HF; New York Heart Association, functional class IV) with a severe alteration of the left ventricular function and an ejection fraction (EF) less than 20%. Their therapeutic treatment included only ACEI and diuretics. The procedures were approved by the Ethical Committee of the University Hospital. The solutions and enzymes used for transportation and dissociation have been detailed previously [13,16–18]. After the enzymatic procedure, the tissue was stored in the same solution as used for rat cells. The myocytes were dispersed by mechanical agitation in the recording chamber just prior to electrophysiological experiments. Only rod shaped, well relaxed and striated myocytes were subjected to experiments.

2.3. Electrophysiological recordings

\( I_{\text{cat}} \) was measured 2 to 10 h after cell dispersion using the whole-cell patch-clamp technique at room temperature (20–22°C). Conditions were optimized to eliminate contaminating voltage-gated inward Na\(^+\) and outward K\(^+\) currents [9,10,13,16]. Bath solutions contained (mmol/l): TEACl (130), CaCl\(_2\) (2), MgCl\(_2\) (1.1), 4-AP (4), HEPES (25), dextrose (22), and phenol red (17.7 mg/l); adjusted to pH 7.4 with TEAOH; 290–310 mOsm/l. To measure the waveforms of Ba\(^{2+}\) currents through Ca\(^{2+}\) channels in the same conditions, 2 mM BaCl\(_2\) replaced CaCl\(_2\). Recording pipettes contained (in mmol/l): CsCl (130), EGTA (10), HEPES (25), Mg-ATP (3), Mg-GTP (0.4); adjusted to pH=7.4 with CsOH; the osmolarity of the buffer was 290–310 mOsm/l. Junction potentials between the intrapipette solution and the reference electrode were cancelled before obtaining the tight seals. Experiments were performed using large low-resistance pipettes (<3 \( \Omega \) when filled with the recording solution). After seal formation (resistance ranging between 1 and 20 GΩ) and membrane disruption, residual series resistance after electronic compensation were \( \approx \)1.2 MΩ and could not introduce major errors of voltage clamp (<4 mV) for currents <2 nA. Whole cell membrane capacitances, determined by integrating the capacitive current recorded during a 5 mV voltage step from a HP of −80 mV, ranged between 60 and 200 pF. The voltage-clamp circuit was provided by an Axopatch 200A patch-clamp amplifier. Experimental parameters, such as holding potentials (HPs) and test potentials were controlled with an IBM PC connected through a Tekmar Labmaster analog interface (Axon instruments, Burlingame, CA, USA) to the electrophysiological equipment. Data acquisition and analyses were performed using the pCLAMP software (Axon instruments). Signals were filtered at 3–5 kHz prior to digitization and
storage. $I_{\text{CaL}}$ was recorded at a test pulse of $-10$ mV (100 ms duration) delivered from a HP of $-80$ mV. A voltage-ramp protocol was also used in some cells. No T-type $I_{\text{Ca}}$ was evident in human atrial and ventricular cells, as well as in rat ventricular myocytes [9,13,16,18]. Although a tetrodotoxin-sensitive low threshold $I_{\text{Ca}}$ flowing through Na⁺ channels was recorded sometimes, this current had no significant contribution here because it is observed only from very negative HPs (it is 95% inactivated at $-80$ mV) and it is small at a test pulse of $-10$ mV [18].

2.4. Analysis of $I_{\text{CaL}}$

The effects of conditioning membrane potentials on $I_{\text{CaL}}$ were examined using the paradigm illustrated in Fig. 1A. Peak $I_{\text{CaL}}$ were measured as the difference between the maximal inward current amplitude at $-10$ and the zero current level. The Ca²⁺ entry was quantified by integrating $I_{\text{CaL}}$ (pA·ms) during the 100-ms duration test pulse, rather than measuring only peak current. The decay of $I_{\text{CaL}}$ was also measured. Inactivation was best fitted by the sum of two exponential components using the formula

$$I_{\text{CaL}} = I_{\text{CaL,(fc)}} \cdot \exp(-t/\tau_{(fc)}) + I_{\text{CaL,(sc)}} \cdot \exp(-t/\tau_{(sc)})$$

where, $I_{\text{CaL,(fc)}}$ and $I_{\text{CaL,(sc)}}$ are respectively the current amplitudes of the fast and slow components of $I_{\text{CaL}}$ and $\tau_{(fc)}$ and $\tau_{(sc)}$ are respectively their related time constants of inactivation. This procedure has been described in detail previously [9,11]. The zero time was set slightly before peak currents to determine $I_{\text{CaL,(fc)}}$ and $I_{\text{CaL,(sc)}}$ and, in all cases, the sum of $I_{\text{CaL,(fc)}} + I_{\text{CaL,(sc)}}$ accounted for peak $I_{\text{CaL}}$.

2.5. Statistical analysis

All averaged or normalized data are presented as mean ±S.E.M. The significance between groups of data was assessed using Student’s $t$ test (for paired and unpaired samples as appropriate). Results were considered not significant (ns) with $P>0.05$, significant (*) with 0.01 < $P<0.05$, very significant (**) with 0.001 < $P<0.01$ and extremely significant (***) with $P<0.001$. Box and whiskers representation was also used as noted (Fig. 7). The box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median (50th percentile). Whiskers extend down to the smallest value and up to the largest (showing the range of data).

2.6. Solutions

Isoproterenol (Iso), serotonin, and dibutyryl-cAMP (db-cAMP) (Sigma) and ryanodine (Biomol) were prepared as

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Fig. 1. Modulation of $I_{\text{CaL}}$ by conditioning potentials in rat ventricular myocytes. (A) Experimental protocol: $I_{\text{CaL}}$ were evoked at $-10$ mV following 5-s conditioning pulses to various potentials. (B) Typical waveforms of $I_{\text{CaL}}$ evoked from $-80$ and $-50$ mV. Note the change in the waveform of $I_{\text{CaL}}$. In the inset, currents have been scaled for direct comparison of their kinetics. (C) Complete representative relationship between normalized Ca entry (quantified by integrating $I_{\text{CaL}}$ (pA·ms) during the test pulse: see Methods) and conditioning voltages in the same cell as in (B). Note the graded increase between $-80$ and $-40$ mV.
1 or 10 mmol/l stock solutions in distilled H2O and subsequently diluted at the desired working concentrations in test solutions. The control and test solutions were applied by using a multiple capillary perfusion system (200 μm inner diameter, flow-rate 0.5 ml/min) placed in close proximity of the cell (<0.5 mm). Each capillary is fed by a reservoir 50 cm above the bath. Rapid and complete solution changes were made by switching the capillary to the next.

3. Results

3.1. Depolarization-induced facilitation of \( I_{\text{Ca,l}} \) in rat ventricular cells

To study the amplitude and the waveforms of \( I_{\text{Ca,l}} \) at various resting membrane potentials, rat ventricular cells were voltage clamped at HP \(-80 \) mV, and subsequently depolarized to various conditioning potentials between \(-80 \) and \(-10 \) mV for 5 s prior to voltage steps to \(-10 \) mV (which activates maximal current amplitude) for 100 ms in duration (Fig. 1A). The 5-s conditioning pulse duration was selected to achieve steady-state conditions since no further changes in \( I_{\text{Ca,l}} \) waveforms were evident during conditioning pulses of longer duration. When this protocol was applied, a facilitation of \( I_{\text{Ca,l}} \) was induced by conditioning pulses of moderate amplitude (Fig. 1B) in 88% of the cells \((n=24)\) leading to a substantial increase of \( \text{Ca}^{2+} \) influx as measured by integrating \( \text{Ca}^{2+} \) entry (see Methods) during the test depolarization (Fig. 1C). This increase resulted mainly from the slowing of \( I_{\text{Ca,l}} \) decay (Fig. 1B). On average, the increase in \( \text{Ca}^{2+} \) entry promoted by depolarizing the conditioning potential from \(-80 \) to \(-50 \) mV was 39±2%. In most cells this depolarization-induced facilitation of \( \text{Ca}^{2+} \) channel activity was still marked at \(-40 \) mV. Further depolarization to voltages less negative than \(-40 \) mV resulted in clear voltage-dependent inactivation of \( \text{Ca}^{2+} \) entry due to the decreased availability of the \( \text{Ca}^{2+} \) channels for opening [4]. No further change of the decay of \( I_{\text{Ca,l}} \) was observed at these voltages (data not shown). However, 12% of the cells lacked the voltage-dependent facilitation of \( I_{\text{Ca,l}} \), which could be related to a distinct physiological state of the cells (e.g. phosphorylation level).

3.2. Modulation by Isoproterenol and dibutyryl-cAMP

We investigated whether the depolarization-induced facilitation of \( I_{\text{Ca,l}} \) is modulated by \( \beta \)-adrenergic receptor stimulation in rat ventricular cells. This model appeared suitable because facilitation was observed in nearly all cells. Extracellular application of Iso, a \( \beta \)-adrenergic agonist, increased the amplitude of \( I_{\text{Ca,l}} \) evoked at \(-10 \) mV by 130±40% \((n=8)\) when applied at its maximally effective concentration \((2 \mu\text{mol/l})\). \( I_{\text{Ca,l}} \) peak amplitude slowly increased and reached a plateau within 30 s to 40 s which reflected the time required to achieve steady-state phosphorylation of \( \text{Ca}^{2+} \) channels by the activated catalytic subunit of protein kinase A [3,4]. We found that \( \text{Ca}^{2+} \) could enhance (when present in control conditions: Fig. 2Aa) or even unmask (when absent: Fig. 2Ab) the depolarization-induced facilitation of \( I_{\text{Ca,l}} \) in seven out of eight cells. The increase of \( \text{Ca}^{2+} \) entry induced by depolarizing the cells from \(-80 \) to \(-50 \) mV during the conditioning prepulse was enhanced by a factor 2 \((87±9\%)\) after maximal stimulation of the \( \text{Ca}^{2+} \) channels by Iso. This enhancement was observed at all test pulses activating \( I_{\text{Ca,l}} \); that is, it did not reflect a shift in the current–voltage relationship as observed using a ramp protocol (Fig. 2B). Fig. 2B also shows that the larger depolarization-induced facilitation of \( I_{\text{Ca,l}} \) in the presence of Iso (compare vertical bars) occurs at all voltages of the current–voltage relationship. Since enhancement of the activity of cardiac \( \text{Ca}^{2+} \) channels following \( \beta \)-adrenergic receptor stimulation occurs mainly via intracellular cAMP production [2–5], we assessed the direct activation of protein kinase A by cAMP. Addition of 100 μmol/l of the membrane permeable analog db-cAMP to the extracellular perfusion solution could mimic the effect of Iso and enhanced facilitation by a factor 2 \((85±16\%; n=4)\). This value was very similar to that obtained for Iso which suggested that cAMP mediates most of the effect of Iso.

3.3. Link with \( \text{Ca}^{2+} \)-dependent inactivation

Depolarization-dependent facilitation reflects a reduction in the fast decay kinetics of \( I_{\text{Ca,l}} \). In various cardiac cells, including human, there is a joint dependence of the inactivation of \( I_{\text{Ca,l}} \) on both voltage and intracellular \( \text{Ca}^{2+} \) [2,4,16,19,20]. In this context, \( I_{\text{Ca,l}} \)-triggered \( \text{Ca}^{2+} \) release from the SR is an important feed-back signal in determining the rate of inactivation of the L-type \( \text{Ca}^{2+} \) channel [20–24]. To examine whether the depolarization-induced change in the decay kinetics of \( I_{\text{Ca,l}} \) depends on the amount of \( \text{Ca}^{2+} \) released from the SR, we used ryanodine to deplete the SR \( \text{Ca}^{2+} \) content and tested the effects. The results showed that ryanodine reduces the rate of inactivation of \( I_{\text{Ca,l}} \) (Fig. 3A). In these conditions, no facilitation could be induced following conditioning depolarizations (Fig. 3B). The increase of \( \text{Ca}^{2+} \) entry that is normally induced by depolarizing the cells from \(-80 \) to \(-50 \) mV was abolished in all of five cells tested. The slow decay of \( I_{\text{Ca,l}} \) was the limiting factor. On average, the potentiation of \( I_{\text{Ca,l}} \) observed during facilitation decreased from 42±7% to 8±1% after ryanodine \((***P<0.001)\). Therefore, we concluded that depolarization-dependent facilitation involves SR \( \text{Ca}^{2+} \) release-induced inactivation of \( I_{\text{Ca,l}} \).

We next examined the effect of replacing extracellular \( \text{Ca}^{2+} \) by \( \text{Ba}^{2+} \), which slows the decay of \( \text{Ca}^{2+} \) channel current, on facilitation to assess the effect of cAMP
Fig. 2. Enhancement of depolarization-induced facilitation of \( I_{CaL} \) by Iso in rat ventricular myocytes. (A), (a) The depolarization-induced facilitation of \( I_{CaL} \), present in control conditions (squares), was enhanced during exposure to Iso (2 \( \mu \)mol/l: circles). \( Ca^{2+} \) entry was quantified by integrating \( I_{CaL} \) during the test pulse. (b) Same experiment as in (a) but facilitation was absent in control. It was enhanced by Iso (2 \( \mu \)mol/l). Typical waveforms of \( I_{CaL} \) evoked from \(-80\) and \(-50\) mV are shown. (B) Same experiment as in (A) but \( I_{CaL} \) was evoked using a voltage-ramp protocol delivered from \(-80\), \(-60\), \(-40\) mV as shown in the left panel. In this myocyte, facilitation was present prior to exposure to the drug. Note the voltage-dependent facilitation at all voltages. Note also the greater facilitation in the presence of Iso and the larger effect of Iso at \(-40\) mV (e.g., compare bars between \(-80\) and \(-40\) mV).

Independently of \( Ca^{2+} \). In these conditions, \( Ca^{2+} \)-dependent inactivation of \( Ca^{2+} \) channel current is completely removed and only purely voltage-dependent inactivation occurs [2,4,9,19]. When the conditioning potential was gradually changed from \(-80\) to \(-50\) mV prior to the test pulse, no increase of current peak amplitude was observed (Fig. 4A, left panel; Fig. 4B). In fact, the peak current decreased gradually and the \( \beta \)-adrenergic receptor stimulation by Iso enhanced this decrease (Fig. 4A, right panel; Fig. 4B) which is related to the leftward shift of the steady-state inactivation curve described before [25]. For example, the percentage of decrease observed for a conditioning prepulse of \(-50\) mV (vs. \(-80\) mV) was enhanced from \(14\%\) to \(23\%\) (\(* P<0.02; n=9\)). These results contrasted markedly with the large increase obtained using \( Ca^{2+} \) as the charge carrier (Fig. 1). Furthermore, analyzing the variations in integrated \( Ca^{2+} \) and \( Ba^{2+} \) entry during the test pulse confirmed that there is no marked increase of \( Ba^{2+} \) currents between \(-80\) and \(-50\) mV (although a small slowing of the overall current decay may occur) and that Iso induces a large decrease (Fig. 4C). These results suggested therefore that facilitation of \( Ca^{2+} \) channel activity does not occur independently of \( Ca^{2+} \) and does not reflect a direct effect of cAMP-dependent phosphorylation on the channels.

3.4. Depolarization-induced facilitation of \( I_{CaL} \) in human atrial cells

Another objective of this study was to investigate whether the depolarization-induced facilitation of \( I_{CaL} \) could be observed in human cardiomyocytes. In human atrial cells, \( I_{CaL} \) started to activate between \(-40\) and \(-30\) and reached maximal peak amplitude between \(-10\) and 0.
Fig. 3. Effect of ryanodine on voltage-dependent facilitation. (A) Typical waveform of $I_{Ca,L}$ evoked at $-10$ mV from a HP of $-80$ mV in control conditions (control) and after extracellular exposure to 1 μM ryanodine. Maximal effect was generally achieved after 10 min. (B) The depolarization induced facilitation of $I_{Ca,L}$ present in control conditions (a) was markedly abolished after steady-state effect of ryanodine (b). Typical waveforms of $I_{Ca,L}$ evoked at $-10$ from $-80$ and $-50$ mV are shown. (C) Bar graphs show the averaged decrease (mean±S.E.M., $n=5$) of $I_{Ca,L}$ facilitation after ryanodine (1 μM/l). The effect was extremely significant (***$P<0.001$).

mV as described before [5,16,17,20]. To study the waveforms of $I_{Ca,L}$ at various conditioning diastolic membrane potentials, cells were voltage-clamped using the protocol employed for rat ventricular cells with a test pulse at $-10$ mV (Fig. 1A). Two types of responses were observed. In some cells, Ca$^{2+}$ influx was increased following moderate pre-depolarizations (Fig. 5A) which resulted mainly from the slowing of current decay. As described in rat cardiomyocytes, the increase was graded with increasing conditioning prepulses between $-80$ and $-40$ mV (Fig. 5A). This behavior, observed in 13 cells, could lead to a substantial increase of Ca$^{2+}$ entry (up to 50%) during the test pulse. In this group, the averaged increase was $22±5\%$ (between $-80$ and $-50$ mV). The Ca$^{2+}$ entry decreased as the conditioning prepulse was further depolarized from $-40$ to 0 mV, due to voltage-dependent steady-state inactivation of Ca$^{2+}$ channels (Fig. 5A). On the other hand, no significant facilitation occurred in 18 other cells in which only the voltage-dependent decrease of Ca$^{2+}$ channels availability for opening was evident between $-50$ and $-10$ mV (Fig. 5B). This cell population was characterized by slow inactivation kinetics as illustrated in
Fig. 4. Effect of conditioning potentials on Ba\(^{2+}\) currents in rat ventricular myocytes. (A) Typical waveforms of Ba\(^{2+}\) currents evoked at −10 mV from various conditioning voltages between −80 and −30 mV in the absence (left panel) and presence (right panel) of Iso (1 μmol/l). Note the decrease in current peak amplitude with little change in the decay kinetics. (B) Bar graphs show the averaged decrease (mean ± S.E.M.) of Ba\(^{2+}\) currents peak amplitude for various conditioning prepulses between −80 and −50 mV in the absence and presence of Iso. The significance is quoted as defined in Methods for each test. (C) Comparison of the variations of Ca\(^{2+}\) and Ba\(^{2+}\) entry (quantified by integrating currents (pA·ms) during the test pulse at −10 mV; see Methods) evoked from conditioning depolarizations at −80 and −50 mV in the absence and presence of Iso. Note the variations in opposite directions.

the inset of Fig. 5B. The superimposition of the two curves suggests that the facilitation phenomenon overlaps with the classical steady-state inactivation of \(I_{\text{CaL}}\) (Fig. 5B). We found no evident morphological difference between cells that exhibited and cells that did not exhibit facilitation.

All human atrial myocytes exhibiting the depolarization-induced facilitation of \(I_{\text{CaL}}\) also exhibited the high-frequency induced facilitation (Fig. 6Aa,b) which we described previously [13,14]. As a corollary, none of the cells lacking the frequency-dependent facilitation exhibited the voltage-dependent facilitation. The two phenomena were not additive; i.e. there was no high frequency-induced facilitation of \(I_{\text{CaL}}\) when the cells were held at a diastolic membrane potential of −50 mV because, as described for
Fig. 5. Modulation of $I_{Ca}$ by conditioning potentials in human atrial myocytes. $I_{Ca}$ were evoked as shown in Fig. 1A. (A) Cell illustrating depolarization-induced increase of Ca$^{2+}$ entry through Ca$^{2+}$ channels in a human atrial cell. Ca$^{2+}$ entry was quantified by integrating $I_{Ca}$ (pA·ms) during the test pulse and normalized. Inset: typical waveforms of $I_{Ca}$ evoked to $-10$ mV from $-80$ and $-50$ mV. (B) Same experiment in another cell with no increase of Ca$^{2+}$ entry. In inset: typical waveforms of $I_{Ca}$ evoked to $-10$ mV from $-80$ and $-50$ mV in this cell population. The superimposed dashed curve was taken from (A).

rat ventricular cells before [11], fast inactivation of $I_{Ca}$ was already greatly reduced at a HP of $-50$ mV (data not shown). Furthermore, as shown above for rat ventricular cells, both types of facilitation were blunted using Ba$^{2+}$ as the charge carrier through the Ca$^{2+}$ channels ($n = 5$; data not shown) because the Ca$^{2+}$-dependent fast decay of the current was dramatically reduced. Finally, as also observed in rat ventricular cells, cAMP-promoting agents enhanced both types of facilitation. This is illustrated (Fig. 6Aa,b) for serotonin (1 μmol/l) which activates the cAMP cascade via the 5HT$_4$-receptor in human atrial myocytes [17]. The membrane permeable analog of cAMP, db-cAMP (100 μmol/l) had similar effects (Fig. 6B). Iso had, on average, more discrete effects (Fig. 6C) owing to some
Fig. 6. Modulation of the depolarization-induced facilitation of $I_{\text{cal}}$ by the cAMP pathway in human atrial cells. (A), (a) The depolarization-induced facilitation of $I_{\text{cal}}$ present in control conditions (squares), was markedly enhanced during exposure to serotonin (1 μmol/l) in a human atrial myocyte. Typical waveforms of $I_{\text{cal}}$ evoked from −80 and −50 mV are shown. In inset (right panel): complete relationship between Ca$^{2+}$ entry and conditioning voltages in the same cell. (b) Enhancement of the high-frequency induced facilitation of $I_{\text{cal}}$ by serotonin in the same cell. The rate of stimulation was increased from 0.1 to 1 Hz. Steady-state was obtained at the fourth stimulation (i.e., within 3 s). In inset (right panel): bars compare Ca$^{2+}$ entry at 0.1 Hz (white bars) and 1 Hz (black bars) in the absence and presence of serotonin. Note the enhancement by serotonin. (B) Enhancement of the depolarization-induced facilitation of $I_{\text{cal}}$ by db-cAMP (100 μmol/l). (C) Bar graphs show averaged enhancements (mean ±S.E.M.) of depolarization-induced facilitation of $I_{\text{cal}}$ by Iso (2 μmol/l), db-cAMP (100 μmol/l) and serotonin (1 μmol/l). The number of experiments for each bar is quoted. Significant statistical differences are indicated as * $P<0.03$. 

variability among cells (two cells lacked facilitation even after Iso) which contrasted with the results obtained in rat ventricular cells. Such variability may result from pathology or drug treatment and, possibly, reflect a down-regulation of β-adrenergic receptors [26,27].

We next investigated the effect of cAMP-promoting agents in further details. Facilitation was clearly linked to the presence of the fast inactivating component of $I_{CaL}$ (Figs. 2Ab and 5A). The decay of macroscopic $I_{CaL}$ was indeed best described by the sum of two exponentials with time constants separated by an order of magnitude (Fig. 7A). They varied between 4 and 17 ms (mean 9±1 ms) for the fast one and between 37 and 200 ms (mean 92±7 ms) for the slow one at a test depolarization of −10 mV as determined in a large population of cells ($n=48$). These time constants underlay two kinetically distinct current components that we will refer to as $I_{CaL(fc)}$ and $I_{CaL(sc)}$. The relative contribution of each component determines the global waveform of $I_{CaL}$ and varies among cells. A double exponential fit to the decay phases of $I_{CaL}$ evoked from each conditioning potential tested provided the amplitudes of $I_{CaL(fc)}$ and $I_{CaL(sc)}$ in each cell. We found that both db-cAMP and Iso, when applied at low rates of stimulation and from HP −80 mV, promoted preferentially $I_{CaL(fc)}$ (Fig. 7B,C). On average, Iso increased the contribution of $I_{CaL(fc)}$ to the global $I_{CaL}$ from 22±4% to 57±4% ($n=30$; ***$P<0.001$). When facilitation was observed, it was related to reduced fast inactivation of $I_{CaL}$ rather than to increased peak current amplitude. This change reflected modifications in the amplitudes of $I_{CaL(fc)}$ and $I_{CaL(sc)}$ with no major changes in $\tau_{(fc)}$ and $\tau_{(sc)}$. The double exponential fits to the decay phases of $I_{CaL}$ revealed that changing the conditioning voltage from −80 to −50 mV resulted in a reduction in the amplitude of $I_{CaL(fc)}$ and a corresponding increase in the amplitude of $I_{CaL(sc)}$. Since there is only a minor change on global peak $I_{CaL}$ amplitude (Fig. 7Da), the changes in $I_{CaL(fc)}$ and $I_{CaL(sc)}$ amplitudes are, therefore, reciprocal and reflect only a change in the inactivation process of the same population of Ca$^{2+}$ channels. $I_{CaL(fc)}$ was predominant (75±3% of total $I_{CaL}$, $n=20$) only in the fraction of cells exhibiting facilitation (Fig. 7Db). Similar analysis of facilitation in rat ventricular cells led to similar conclusion [9,11]. It should be noted that, in contrast, the inactivation of Ba$^{2+}$ currents and Ca$^{2+}$ currents in the presence of ryanodine could be best fitted by a single exponential corresponding to the slower component of $I_{CaL}$ (data not shown).

### 3.5. Facilitation in failing hearts

We investigated facilitation in myocytes isolated from failing hearts of transplant recipients. These patients had drug treatment limited to ACE inhibitors and to diuretics (no Ca$^{2+}$ channel antagonist, no β-adrenergic blocker). Fig. 8A shows that facilitation was impaired in most atrial myocytes isolated from end-stage HF patients with a low ejection fraction (<20%) as compared to cells isolated from the non-HF patients with an ejection fraction >40%. On average, facilitation decreased from $10\pm2\%$ ($n=31$) to $-2\pm3\%$ ($n=11$; ***$P<0.001$). Both the β-adrenergic- and the 5HT$\_4$-receptors stimulations were inefficient to induce significant facilitation (ns, $P>0.05$) though large effects could be observed in a few individual cells. Facilitation was also almost absent in HF ventricular cells (Fig. 8B). Although Iso was able to promote very substantial facilitation (>50%) in three cells, the averaged effect was below significance at the cell population level. These findings contrasted markedly with observations made in rat ventricular cells in which Iso enhanced facilitation. We determined that, the slow component of inactivation $I_{CaL(sc)}$ was the predominant component of $I_{CaL}$ in human HF cells lacking facilitation (data not shown). Therefore, facilitation is not observed in HF mainly because the decay kinetics of $I_{CaL}$ are slower than in normal cells.

### 4. Discussion

In the present study, we emphasize that variations of the diastolic membrane potential at physiological range regulate the decay kinetics of $I_{CaL}$ and thereby, control transmembrane Ca$^{2+}$ entry into cardiomyocytes. The new findings are: (i) depolarization-dependent regulation of $I_{CaL}$ decay kinetics occurs not only in rat but also in human cardiomyocytes; (ii) it is the basis of the phenomenon referred to as facilitation; (iii) facilitation reflects removal of the SR Ca$^{2+}$ release-induced inactivation of $I_{CaL}$ during depolarization and is favored by intracellular cAMP, presumably via enhanced SR Ca$^{2+}$ load; and, (iv) facilitation is defective in cardiomyocytes isolated from end-stage failing human hearts.

#### 4.1. Depolarization-induced facilitation of $I_{CaL}$

A well-known effect of conditioning depolarizations between −50 and 0 mV is to decrease peak $I_{CaL}$ amplitude due to a voltage-dependent decrease in the availability for opening of the Ca$^{2+}$ channels [4]. However, conditioning depolarizations between −80 and −50 mV do not decrease but instead increase gradually Ca$^{2+}$ influx during the test depolarization. This type of facilitation evidenced initially in rat ventricular cells [9,11,28] is shown here for the first time in human cardiomyocytes. As described before for the high-frequency induced facilitation [6–15], it is related mainly to a marked slowing of $I_{CaL}$ decay kinetics. The slight increase of $I_{CaL}$ peak amplitude may result only from slower inactivation rather than from an increase in the number of Ca$^{2+}$ channels available for opening [11]. It has been widely reported that facilitation reflects a genuine effect on Ca$^{2+}$ channels [6–15,29]. Facilitation is associated with an inward current both at negative and positive voltages [29]. It seems thus unlikely
Fig. 7. Link of the depolarization-induced facilitation to cAMP and fast decay kinetics of $I_{Ca}$ in human atrial cells. (A) The decay of $I_{Ca}$ was best fitted by two time constants. The box and whiskers representation (see Methods) shows that the fast and slow time constants are separated by an order of magnitude. Analysis was performed on 48 cells (***, $P < 0.001$). (B) Illustration of the preferential increase of $I_{Ca,fc}$ (fc for fast component; sc for slow component) by db-cAMP (100 μmol/l). The time constants $\tau_{fc}$ and $\tau_{sc}$ were respectively 5 and 110 ms (for both traces). (C) Preferential increase of $I_{Ca,fc}$ by Iso (2 μmol/l). (a) Illustration for a single cell. $\tau_{fc}$ and $\tau_{sc}$ were respectively 5.3 and 70 ms (for both traces). (b) Box and whiskers representation of the effect of Iso on the contribution of $I_{Ca,fc}$ to $I_{Ca}$. Analysis was performed on 30 cells (***, $P < 0.001$). (D), (a) Depolarization-induced interconversion between $I_{Ca,sc}$ and $I_{Ca,fc}$. The experiment was performed using the protocol shown in the inset in the presence of 1 μM Iso. The conditioning potential at $-80$ mV induced preferential activation of $I_{Ca,sc}$ whereas the conditioning potential at $-50$ mV promoted mainly activation of $I_{Ca,fc}$. The observed voltage-dependent changes in $I_{Ca,fc}$ and $I_{Ca,sc}$ were reciprocal. $\tau_{fc}$ and $\tau_{sc}$ were fixed at respectively 8 and 120 ms (for both traces). (b) Box and whiskers representation of the contribution of $I_{Ca,fc}$ to global $I_{Ca}$ in cells exhibiting facilitation (F) or no facilitation (NF) either in the presence or absence of Iso. Analysis was performed on 20 and 25 cells respectively. Results were extremely significant (***, $P < 0.001$).
that fast Ca\(^{2+}\)-activated chloride currents have a contribution because these currents should be in opposite directions (inward at negative voltages and outward at positive potentials) due to the use of symmetrical chloride concentrations [30].

4.2. Links with intracellular cAMP and Ca\(^{2+}\)

The early fast decay of \(I_{\text{Ca,L}}\) and its related component \(I_{\text{Ca,L(sc)}}\) are determined by a Ca\(^{2+}\) release-induced inactivation in a microdomain inaccessible to EGTA and not by its own influx [2,20–24]. This local Ca\(^{2+}\) signalling can contribute to 65–75\% of inactivation [22,23] and has been demonstrated using ryanodine or thapsigargin which both abolish Ca\(^{2+}\) release from the SR or, yet, phospholamban-deficient mice [15,20,22–24]. Our result showed that when the SR-Ca\(^{2+}\) release and thereby the fast Ca\(^{2+}\)-dependent inactivation of \(I_{\text{Ca,L}}\) are reduced by ryanodine, no depolarization-induced facilitation occurs. Therefore, the slow inactivation of \(I_{\text{Ca,L}}\) which underlies the depolarization-induced facilitation, involves reduced Ca\(^{2+}\) release-induced inactivation as demonstrated for frequency-dependent facilitation [7,8,15].

The primary effect of cAMP is to increase peak \(I_{\text{Ca,L}}\) due to both an increase in probability of Ca\(^{2+}\) channel opening as well as an increase in the number of functional Ca\(^{2+}\) channels [2–4]. We show here that another effect of cAMP is to enhance the depolarization-induced facilitation of \(I_{\text{Ca,L}}\). Our detailed analysis reveals that facilitation occurs because the fraction of current exhibiting fast Ca\(^{2+}\)-dependent inactivation (\(I_{\text{Ca,L(fc)}}\)) is enhanced by cAMP. Therefore, there is a greater proportion of channels that can switch from fast to slow inactivation, with \(I_{\text{Ca,L(sc)}}\) becoming the major current component during facilitation. Consistently, when the Ca\(^{2+}\)-dependent inactivation is abolished using Ba\(^{2+}\) as the permeating ion, \(\beta\)-adrenergic receptors stimulation is unable to promote facilitation. Thus, we conclude that the effect of cAMP-dependent phosphorylation on facilitation is not direct and is related to enhanced Ca\(^{2+}\)-dependent inactivation of \(I_{\text{Ca,L}}\) resulting from stimulated Ca\(^{2+}\) load of the SR.

In contrast with the high-frequency induced facilitation, the depolarization-induced facilitation occurs in a range of conditioning voltages considered too negative (\(< -50\) mV) to activate significant macroscopic \(I_{\text{Ca,L}}\). Two mechanisms could account for this intriguing phenomenon. Voltage-driven Ca\(^{2+}\) release from the SR [31] or alternatively, Ca\(^{2+}\) entry via a very small number of Ca\(^{2+}\) channels with a high driving force for Ca\(^{2+}\) can generate large localized Ca\(^{2+}\) concentrations and, hence, SR-Ca\(^{2+}\) release. Interestingly, cAMP-dependent phosphorylation induces a leftward shift of the voltage threshold required for channel activation and may favor Ca\(^{2+}\) entry at potentials as negative as \(-60\) mV [25,32]. The precise mechanism(s) determining the coupling between the conditioning depolarization and Ca\(^{2+}\) release-induced inactivation of \(I_{\text{Ca,L}}\) will be worth studying in the future.

4.3. Possible physiological relevance

Another form of depolarization-induced facilitation of \(I_{\text{Ca,L}}\) has been described in cardiac cells. In this case, strong conditioning depolarizations (\(>40\) mV) increase Ca\(^{2+}\) channels activity when Ba\(^{2+}\) is the permeating cation [25,33]. In contrast, facilitation here only requires moderate depolarization but Ca\(^{2+}\) is mandatory. The slowing of \(I_{\text{Ca,L}}\) inactivation associated with reduced SR Ca\(^{2+}\) release,
may supply additional Ca\(^{2+}\) ions for contraction and loading of the SR [34,35]. It may be an adaptive mechanism to support the increase in cardiac contraction during exercise and stress in response to catecholamines release [36]. For example, β-adrenergic receptors stimulation has been reported to induce cell depolarization in guinea-pig papillary muscles and sheep Purkinje fibers [37–39].

Activation of chloride currents and ‘pacemaker’ current \(I_f\) is also expected to induce cell depolarization [40,41], thus favouring \(I_{Ca}\) facilitation. However, any pathological situation generating ectopic beats and cell depolarization may contribute to the arrhythmogenicity of Ca\(^{2+}\) L-type channels activity. This may be consistent with the slow decay kinetics of \(I_{Ca}\) that we observed here in HF myocytes. This may be consistent with the recent single channel data showing that L-type Ca\(^{2+}\) channels from failing hearts remain available for a longer time during depolarization [48]. Lower intracellular cAMP, resulting from the down-regulation of β-adrenergic receptors [22,23] or from an increase in G-protein inhibition [49], may also contribute to lessening of current inactivation and lack of facilitation due to inefficiency to stimulate the SR-Ca\(^{2+}\)-ATPase activity [24].

In conclusion, we show that facilitation of L-type Ca\(^{2+}\) channels activity can be induced by moderate depolarization of the diastolic membrane potential in cardiomyocytes. This process may have profound physiological relevance for catecholamines enhancement of Ca\(^{2+}\) influx. Facilitation is impaired in human HF, possibly due to both low intracellular cAMP (low opening probability of the Ca\(^{2+}\) channel) and altered re-uptake of intracellular Ca\(^{2+}\) by the SR which decreases the negative feed-back by the Ca\(^{2+}\) released from the SR on Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels.

Acknowledgements

We thank Matteo Elia Mangoni, Steve Dubel and Govindan Dayanithi (Montpellier, F) and Jamie Vanden-berg (Cambridge, UK) for improving the manuscript. This work was supported by grants from the ‘Association Recherche et Partage’ (to SR), the ‘Association Française contre les Myopathies’ and laboratoires Servier (SBL).

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